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13. ABSTRACT (Maximum 200) The primary objective of experiments carried out during the second year focused on determining the behavioral significance of neuroprotective effects achieved by systemic hypothermia following moderate spinal cord injury. In these experiments moderate hypothermia was initiated 30 minutes post-injury for a period of four hours. Two days post-injury we initiated the behavioral assessment of locomotor function. In anticipation of future therapeutic applications of combined hypothermia and pharmacological treatment protocols, a second purpose of experiments during the second year was to evaluate the morphological and behavioral effects of an NMDA antagonist and nitric oxide synthase inhibitor (agmatine) following traumatic spinal cord injury. The major findings of these studies have shown that significant differences are observed in the behavioral assessment scores of animals undergoing hyperthermia compared to animals receiving normothermic treatment. Similarly, significant differences were observed following systemic administration of agmatine for 14 days post-injury. Unfortunately, no synergistic or additive effects were achieved when agmatine and hypothermia were combined. Overall, the results support the original hypothesis of this proposal that whole body hypothermia is capable of producing enhanced functional recovery following traumatic spinal cord injury.	
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FOREWORD

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ANNUAL REPORT (1999)

THERAPEUTIC HYPOTHERMIA FOLLOWING TRAUMATIC SPINAL INJURY

ROBERT P. YEZIERSKI, PH.D.

INTRODUCTION

The research carried out during the second year of funding focused on the effects of systemic hypothermia alone and in combination with the endogenous neuroprotective agent agmatine on morphological and behavioral outcome measures following traumatic spinal cord injury. The discussion below describes the subject, purpose and scope the research and the background of previous work related to: (a) the neuroprotective effects of hypothermia in models of CNS trauma and ischemia; (b) recent studies supporting the beneficial effects of modest cooling (1-5°C) in CNS injury; (c) the applicability of (a-b) to traumatic spinal cord injury (SCI); and (d) the neuroprotective effects of agmatine.

(a) Hypothermia and CNS Protection: The premise that lowering CNS temperature protects against the detrimental effects of hypoxia and ischemia evolved in the 1950's. From a need to protect nervous tissue during vascular operations where circulation to the brain and/or spinal cord was interrupted hypothermia emerged as an adjunct to conventional therapeutic interventions. The protective influence of moderate and severe hypothermia was first demonstrated in experimental models of both spinal and cerebral ischemia (Beattie et al., 1953; Pontius et al., 1954, 1955; Marshall et al., 1956; Rosomoff, 1954, 1959), and its beneficial effects were concluded to be secondary to the lowering of cerebral metabolic demands. In early studies Rosomoff (1959) described a canine model of middle cerebral artery occlusion where hypothermic treatment (22-24°C for 1 hour) fifteen minutes after ischemia was protective against neurologic injury and death. Similarly, tolerance to interruption of cerebral circulation in dogs was dramatically increased (threefold) when body temperature was reduced to 23°-26°C (Marshall et al., 1956). Similar observations have been reported in primates subjected to prolonged periods (45-75 minutes) of cardiac arrest (Kopf et al., 1975). These observations lead to the utilization of CNS cooling, albeit on a limited scale, as a form of treatment in pathological conditions such as stroke and spinal cord trauma.

Historically, methods for lowering CNS temperature to protect against the detrimental effects of hypoxia and ischemia were based on the observation that hypothermia reduces CNS metabolic activity and cerebral metabolic rate for oxygen. These effects decrease energy requirements of tissue and increase the period it can survive in an energy deficient state (Rosomoff and Holaday, 1954; Hägerdal et al., 1975, 1978; Kramer et al., 1968, Michenfelder and Theye, 1970). Hypothermia has also been shown to protect against loss of phosphocreatinine and the accumulation of lactate and NADH following cerebral hypoxia (Michenfelder et al., 1976; Hägerdal et al., 1978). In early studies hypothermia, for example, was found to decrease cerebral blood flow and oxygen consumption proportionally from 25°C to 35°C (Rosomoff and Holaday, 1954; Hägerdal et al., 1975). Systemic hypothermia was also shown to significantly lower the rate of cerebral ATP depletion following interruption of cerebral circulation (Michenfelder and Theye 1976, Kramer et al. 1968). Other beneficial actions of hypothermia include attenuation of edema and hemorrhage formation that occurs in SCI (Green et al., 1973) and the possible dialysation of toxins secondary to perfusing the cord with hypothermic solutions (Tator and Deecke, 1973). Recently, Tuzgen et al. (1998) reported that epidural cooling with chilled saline for 30 minutes following traumatic SCI significantly reduced secondary tissue damage due to the peroxidation of lipid membranes.

Neuroprotective Effects of Local Spinal Cord Cooling: Based on early studies involving brain injury, hypothermic techniques were modified to provide local cooling to the injured spinal cord. The technique was first successfully applied to experimental spinal cord injury by Albin et al. (1965) who perfused the traumatically injured spinal cord of dogs with cold isotonic saline (5°C) for 2.5 hours post-injury. As a result of this treatment there was a dramatic recovery of neurologic function compared to animals without this treatment. These investigators subsequently demonstrated beneficial effects of local spinal cord cooling (LSCC) in a similar SCI model in monkeys, even when the application of LSCC was delayed for four hours after injury (Albin et al., 1968). Following these observations, numerous experimental SCI studies ensued where animals were successfully treated with local hypothermia (Albin et al., 1965, 1968; Ducker and Hamit, 1969; Kelly et al., 1970; Black and Markowitz, 1971; Tator and Deecke, 1973; Campbell et al., 1973; Hansebout et al., 1975; Kuchner and Hansebout, 1976; Eidelberg et al., 1976; Wells and Hansebout, 1978).

In the assessment of beneficial effects of LSCC following SCI a variety of outcome measures have been used: (a) evoked potentials (Thienprasit et al., 1975); (b) degree of hemorrhage and edema formation (Green et al., 1973); (c) motor performance (Hansebout et al., 1975; Ducker et al., 1969; Thienprasit et al., 1975); and (d) histopathological analysis (Green et al., 1973). In many of these experiments LSCC was achieved via perfusion with a cold solution or an epidural heat exchanger and investigators aimed at achieving extremely low temperatures (in the neighborhood of 10-15°C below normal). In these studies spinal cord cooling following injury was found to be neuroprotective even if its application was delayed three to six hours post injury (Albin et al., 1968; Ducker and Hamit, 1969; Kelly et al., 1970; Thienprasit et al., 1975; Wells and Hansebout, 1978). Furthermore, the optimal duration of treatment was reported to be approximately four hours (Wells and Hansebout, 1978). Negative studies of LSCC following traumatic SCI have also been noted (Black and Markowitz, 1971; Howitt and Turnbull, 1972; Eidelberg et al., 1976). It should be emphasized, however, that differences in outcome may be secondary to differences in experimental design among different investigators including: (1) animal species; (2) anesthetic regime; (3) method of injury; (4) administration of other drugs; (5) opening the dura; and (6) techniques used to cool the cord. Additionally, spinal cord temperatures were not monitored in a similar fashion in all studies. Some investigators reported only epidural temperatures, and in large species the temperature gradient from epidural to the anterior column can be significant (Wells and Hansebout, 1978). In spite of the inconsistency of experimental design, lack of proper controls, and variability of injury models, most experimental data strongly support the beneficial effects of LSCC in experimental SCI.

The favorable results in animal experiments led to a limited number of cases where local cord cooling was used in human SCI patients (Selker, 1971; Meacham and McPherson, 1973; Koons et al., 1972; Negrin, 1975; Bricolo et al., 1976; Tator, 1979; Hansebout et al., 1984). The results, however, have been difficult to interpret for a variety of reasons: (1) most investigators report only a small number of cases; (2) controls have not been used in any series; (3) variability in level of injury; (4) results have been generally reported as the number of patients that improved or regained function as opposed to utilizing formal grading methods for measuring outcome; (5) the time interval from injury to application of cooling and duration of treatment have been highly variable; (6) a combination of different drug treatments (usually steroids) have been utilized in conjunction with LSCC; and (7) medical causes of spinal compression other than acute SCI, have been included in some studies. In spite of these complications Hansebout et al. (1984) in reviewing the application of this technique to humans concluded that the results were encouraging. The application of the technique, however, is fraught with technical and logistical difficulties not to mention the clinical challenge of performing a multilevel laminectomy on medically compromised patients (frequently with multiorgan trauma) while trying to minimize the time interval between injury and the application of cord cooling. These obstacles could be overcome if only modest systemic cooling was required in order to produce neuroprotective effects.

The largest patient series using hypothermia was reported by Meacham and McPherson (1973), in which 14 spinal injured-patients were treated with LSCC. Successful initiation of LSCC within 8 hours or less from the time of injury was achieved in all cases and the authors reported return of function in 7/14 patients. A major concern with this study, however, was a mortality rate of 29%, which the authors attributed to the frequency of respiratory complications that occur in cervical injuries. It is difficult to draw definitive conclusions regarding outcome from this study since controls were not utilized and the period of follow-up was not mentioned. Only two other studies contained more than a small number of SCI patients. The first was a series of 11 patients reported by Tator (1979) and the second was a series of 10 patients reported by Hansebout and colleagues (1984). Both studies found that LSCC provided functional recovery in 27% and 43% of patients respectively, which was considered to be higher than would be expected following conventional treatment.

(b) Mild Hypothermia In CNS Ischemia: The observation that modest hypothermia is neuroprotective in CNS ischemia was first demonstrated by Berntman et al. (1981) who observed that 1-5°C decreases in body temperature diminished the loss of ATP and phosphocreatinine, and lessened the degree of brain tissue acidosis following brain hypoxia. Recent studies in which actual brain temperature was monitored strongly suggest that a drastic lowering of CNS temperature is unnecessary to significantly reduce the degree of tissue damage occurring after brain ischemic injury. It has been documented that modest temperature changes (1-5°C) in models of brain ischemia and trauma can significantly alter the extent of neuronal injury, free radical activity and blood-brain barrier alterations (Busto et al., 1987; Dietrich et al., 1990; Dietrich et al., 1991, Globus et al., 1995). Furthermore, modest brain hypothermia reduces the release of neurotransmitters, such as glutamate, which can mediate secondary injury processes (Busto et al., 1989). These findings suggest that only modest changes in spinal cord temperature may be needed to lessen the extent of tissue injury following trauma. Consistent with this hypothesis are results showing systemic hypothermia lessens the neurological deterioration resulting from brain trauma (Bramlett et al., 1995) and improves neurological outcome following ischemia of the rat spinal cord (Robertson et al., 1986).

(c) The Application of Moderate Hypothermia in SCI: Based on the findings described above, it was hypothesized that mild changes in cord temperature could affect the extent of injury occurring in spinal cord trauma. The rationale for this hypothesis centered around the fact that: (1) local spinal cord cooling has been shown to be beneficial; (2) modest decreases in CNS temperature are effective in models of cerebral and spinal ischemia; and (3) modest decreases in brain temperature in models of CNS ischemia protect against processes which have been implicated in the pathophysiology of SCI, e.g. alterations in the blood-brain barrier, edema formation, production of leukotrienes, and release of neurotoxic substances such as glutamate and aspartate (Dempsey et al. 1987, Dietrich et al. 1990a,b, Busto et al., 1989).

The above observations raised the possibility that modest changes in spinal cord temperature may lessen the extent of tissue injury following trauma. Supportive of this idea Tator and Deecke (1973) reported normothermic perfusion to be as effective as hypothermic perfusion in experimental SCI of moderate severity. The actual temperature of the perfusing solution in these experiments was 36°C, which is mildly hypothermic. Low grade hypothermia is also known to spare ATP and phosphocreatinine concentrations, and decrease the magnitude of lactate accumulation after cerebral hypoxia (Berntman et al., 1981). Finally, systemic hypothermia has been shown to be neuroprotective in an experimental model of spinal ischemia (Robertson et al., 1986). Total body hypothermia in fact has been shown to be beneficial and is the standard of prophylaxis against ischemic SCI during aortic cross-clamping and cardiothoracic procedures involving controlled cardiac arrest. Its implementation in the treatment of brain injury in large trauma centers has been promising. Once parameters for optimal efficacy have been established in spinal injury, it seems logical that clinical trials in spinal cord hypothermia will offer similar rewards. If hypothermia does provide important clinical benefits, it is of equal importance to the spinal

injured patient to determine whether elevated systemic temperatures, as might be experienced during an episode of post traumatic fever, exacerbate the injury process and are detrimental to the recovery of function.

To investigate these hypotheses a preliminary study of modest hypothermia in a weight drop model of SCI in the rat was carried out (Martinez and Green, 1992). In this study female Sprague-Dawley rats (250-300g) were subjected to a 50 gram-centimeter (10 gram weight dropped 5cm) lesion at T8 under halothane-nitrous oxide anesthesia. Epidural temperature was maintained at 33°C in the first group of animals (n=3), and at 37°C in a second group (n=3). These temperatures were achieved by lowering systemic (rectal) temperature to 31-32°C in the hypothermic animals or raising systemic temperature between 38°C-39°C in normothermic animals. The epidural temperature in each group was maintained for four hours post trauma. Following injury, animals were kept under nitrous oxide anesthesia until the termination of the four hour treatment period. Three days post-injury animals were sacrificed and the spinal cords removed for histological examination. All animals remained completely paraplegic during this observation period. Morphological evaluation at the epicenter of lesion sites, however, revealed that the 38°C animals had significantly more hemorrhage and parenchymal damage than the 32°C animals (Martinez and Green, 1992).

In conclusion, local spinal cord cooling has been shown to be effective in the treatment of experimental SCI. Similar beneficial results have been reported in some clinical studies, but the number of patients is small and controls have not been utilized. In addition, the high mortality reported in some studies remains a major concern with its clinical application. Recent findings of the neuroprotective effects of modest hypothermia in brain ischemia, however, may be applicable to SCI and offer a treatment protocol with fewer complications. Indeed, preliminary observations suggest that modest temperature changes, such as can be produced via systemic hypothermia, can affect the degree of tissue injury following spinal trauma (Martinez and Green, 1992). The importance of such findings is that, compared to LSCC, systemic hypothermia provides a much simpler approach by which the cord can be "cooled" and thus obviates the need for acute surgical intervention. If effective, modest systemic hypothermia would provide an additional therapeutic approach that could be applied to the clinical treatment of acute SCI. Because of the effects on metabolic processes it is possible that mild hypothermia could extend the window of therapeutic opportunity for additional pharmacological interventions. Of equal importance hypothermia could be used in neurosurgical procedures of the spinal cord and vascular surgical procedures in which spinal cord perfusion may be compromised. Based on the above discussion it can therefore be concluded that there is sufficient justification in both the scientific and clinical literature for additional studies related to better defining the optimal parameters for the hypothermic treatment of the injured spinal cord.

(d) Neuroprotective Effects of Agmatine: The initial trauma induced by injury together with the complex cascade of secondary events following injury determines the degree of total tissue damage and the ultimate neurological outcome following SCI. These events include microvascular alterations, inflammatory processes, alterations of the biochemical environment, free radical formation, ischemia, and cell injuries (Anderson and Hall, 1993; Lipton and Rosenberg, 1994; Tator and Fehlings, 1991; Li et al., 1996). Presently, numerous agents are proposed to be neuroprotective against CNS injury (for recent reviews see McIntosh, 1993; Mattson and Scheff, 1994; Mocchetti and Wrathall, 1995). Steroids, neurotrophins, cytokines, and gangliosides have been demonstrated to promote neuronal survival or support neuronal growth in various *in vitro* systems (Mattson and Scheff, 1994; Blottner and Baumbarten, 1994; Olson et al. 1994.). Methylprednisolone improves neurological recovery when given early after human SCI (Bracken et al., 1990). Recently, neurotrophins and have also been utilized in several disease models: glial cell-derived neurotrophic factors (GDNF) in Parkinson's disease, nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) in Alzheimer's disease, and insulin-like growth factor (IGF-1) in multiple sclerosis (Hefti, 1997) have been used as neuroprotective agents in an effort to combat the neurodegenerative etiology of these diseases. The neurotrophic factor basic fibroblast growth factor (bFGF) has also been reported to be neuroprotective in models of cerebral ischemia and traumatic brain injury (Koketsu et

al., 1994; Fisher et al., 1995; Dietrich et al. 1996) and to protect neurons from axotomy-induced death (Peterson et al., 1996). In a model of spinal cord compression, bFGF administered locally at the site of lesion was reported to improve hindlimb function in combination with methylprednisolone infusion (Baffour et al., 1995). Recently Teng et al. (1997) reported basic and acidic FGF to be neuroprotective for cholinergic neurons following contusion injury in the rat. In a study that was initiated during the first year of funding and completed during the second year we found that several neurotrophins and growth factors exhibited neuroprotective effects when delivered to the site of traumatic spinal cord injury (Lee et al., 1999).

In view of the long term desire to combine treatment modalities, e.g. hypothermia and pharmacological, we have continued to evaluate the neuroprotective properties of different pharmacological agents. During the past year we have successfully demonstrated the neuroprotective effects of the decarboxylated arginine compound agmatine (Brewer et al., 1998) in an excitotoxic model of spinal cord injury. Since the discovery of agmatine a number of researchers have investigated the physiological role of agmatine in the CNS, e.g. where it is located, sites of action, and physiological role. Agmatine is a naturally occurring substance that is thought to be an endogenous neuroprotective transmitter. It is detectable in rat hippocampus, thalamus, hypothalamus, neocortex, ventral tegmental area and in the periaqueductal region of the midbrain (Otake et al., 1998). Agmatine is detectable in astrocytes (Regunathan et al., 1995) and neurons (Reis and Regunathan, 1998). Agmatine binds with high affinity to $\alpha 2$ adrenergic receptors (AR) (Pinthong et al., 1995) and to the putative imidazoline receptor (Li et al., 1994). Agmatine inhibits inducible NOS (iNOS) (Auguet et al., 1995), nNOS and eNOS (Galea et al., 1996) and antagonizes the NMDA receptor (Yang and Reis, 1996, 1999). For these reasons we have been interested in the use of agmatine as a neuroprotective agent against the excitotoxic component of the secondary injury cascade following primary injury to the spinal cord. Supportive of the neuroprotective properties of agmatine are studies showing that agmatine has protective effects in rodent models of neurotoxic and ischemic brain injury (Gilad et al., 1995).

HYPOTHESES AND TECHNICAL OBJECTIVES

The experiments proposed in the original proposal were aimed at addressing a number of interrelated hypotheses focusing on defining the optimal hypothermic parameters required to produce neuroprotection and behavioral recovery in the injured spinal cord.

- 1. Hypothesis:** There is an interdependent relationship among systemic, epidural, and spinal cord temperatures that can be defined in order to determine the degree of systemic and/or epidural temperature required to produce neuroprotection in the injured spinal cord (Experiment 1, original proposal).
- 2. Hypothesis:** Due to the influence of temperature on a wide range of important homeostatic mechanisms necessary for maintaining the structural and functional integrity of spinal tissue, it is proposed that increases in systemic or site of injury temperatures (hyperthermia) will accelerate the injury process and compromise behavioral recovery, whereas reducing the temperature (hypothermia) will result in neuroprotection and enhanced behavioral recovery (Experiment 2, original proposal).
- 3. Hypothesis:** There is an optimum time (post-injury) when post traumatic hypothermia of injured tissue produces the greatest benefit and is most effective in reducing behavioral deficits and morphological damage (Experiment 3, original proposal).
- 4. Hypothesis:** There is an optimum duration of hypothermic treatment which results in the greatest benefit to neurologic outcome (Experiment 4, original proposal).
- 5. Hypothesis:** Combining hypothermia with a pharmacological treatment will result in an additive or synergistic effect on morphological and behavioral outcome measures (Experiment 5, original proposal).

MILITARY BENEFIT OF PROPOSED STUDIES: If modest decreases in spinal cord temperature, achieved by systemic hypothermia or local cord cooling, are capable of reducing the degree of tissue damage following SCI, such a finding would have a significant impact on current protocols used in the treatment of acute spinal cord injury. The military importance of such a finding relates to the fact that in combat situations there are a limited number of options available for the treatment of spinal injured soldiers. If cooling the spinal cord is a viable treatment option the ability to cool the cord using transcutaneous or systemic hypothermia would provide a much simpler approach in order to gain the benefits of hypothermic neuroprotection. If effective, modest systemic hypothermia would provide a therapeutic treatment which could be applied to the human condition at times when surgical intervention is logistically difficult or when multiple injuries make other modes of treatment difficult to implement without severely compromising the survival of the patient. Conversely, it is important to determine the detrimental effects of elevated systemic and cord temperatures in exacerbating the injury process. Since fever secondary to pulmonary compromise and stressed immune function is a common finding in traumatically injured patients, it is important to establish the clinical consequences of modest elevations in systemic temperature (in terms of augmented tissue damage). This information is especially important in the design of treatment protocols for soldiers that develop febrile conditions post injury.

STATEMENT OF WORK: SECOND YEAR

During the second year of funding one of the primary goals was to establish a therapeutic relationship between spinal cord temperature and behavioral outcome measures following traumatic spinal cord injury (SCI). These studies were intended to extend those carried out during the first year in which it was shown that moderate hypothermia delivered for a period of four hours was neuroprotective following moderate, but not severe, traumatic SCI. Although local spinal cord cooling has been attempted as a form of treatment in experimental and human SCI, most studies have focused on temperature shifts in the range of 15-20°C. Because of the technical difficulties required to achieve these conditions the application of hypothermia as a therapeutic intervention in SCI has been difficult to implement. Recent experimental data, however, suggests that modest changes (1-5°C) in central nervous system (CNS) temperature may positively influence outcome following CNS injury. The specific aim for the past year was, therefore, intended to utilize an established model of contusive spinal injury, i.e. weight drop, and evaluate the effects of systemic hypothermic treatment on morphological and behavioral endpoints following injury. Since we would like to ultimately evaluate the neuroprotective effects of combination therapies, e.g. hypothermia with pharmacological treatments, we also initiated a study to evaluate the neuroprotective effects of the endogenous neuroprotective agent agmatine, both alone and in combination with hypothermia. As described above agmatine has been shown to have neuroprotective properties in models of traumatic and ischemic head injury and excitotoxic spinal cord injury.

1. Based on observations that slight decreases in brain temperature can significantly improve neurologic outcome following ischemic or traumatic brain injury, it was hypothesized that modest decreases (1-5°C) in spinal cord temperature would provide neuroprotection and enhanced behavioral recovery following traumatic spinal injury. In experiments carried out during the first year, we determined that lowering the epidural temperature to 32°C for a period of four hours post injury produced significant neuroprotection within the injured cord. During the second year we extended this research by evaluating the effects of hypothermia on functional outcome.

2. Determine the effects of combining the most beneficial hypothermic regime with a protocol of pharmacological treatment. In these experiments we used the temperature of 32°C, the application time of 30 minutes post injury, and a duration of four hours in combination with systemically delivered agmatine (100mg/kg) and evaluated behavioral and morphological endpoints.

EXPERIMENTAL DESIGN AND METHODS

General Methods

Experimental models of spinal trauma: Although many models have been developed for the production of spinal cord injuries in animals, no single model can perfectly mimic the human condition. Several models, such as the weight drop or Allen (1911) technique and the aneurysm clip compression technique, have been well characterized and documented to create graded, reproducible, spinal cord injuries in rats (Rivlin and Tator, 1978, Gale et al., 1985, Wrathall et al., 1985, Noble et al., 1985). The weight drop method involves dropping a known weight (usually 10g in rodents) a selected distance. As the height of the drop is increased, more severe injuries are produced. In spite of requiring a laminectomy and several reports of it yielding variable neuropathologic changes, the weight drop model remains an accepted standard technique which closely mimics the biomechanics of the human injury and produces injuries which are morphologically similar to those seen in humans (Jellinger, 1976). Furthermore, it has been shown that under careful control of experimental variables, this model will yield reproducible graded injuries (Gale et al., 1985, Wrathall et al., 1985, Noble et al., 1985). In studies being carried out in hypothermia Research Plan injuries are produced using a weight drop device obtained from Dr. Wise Young at New York University. This device is presently being used to produce a standard injury in a multi-center study designed to evaluate the clinical efficacy of drug actions in the treatment of acute spinal cord injury. The injury model to be used is therefore one that has widespread use in the field of spinal cord injury. This feature offers significant advantages, i.e. standardization, when comparing results obtained with different putative therapeutic interventions, e.g. hypothermia versus drug treatments.

Surgical Preparation: Anesthetized adult (250-275g) female Sprague-Dawley rats had the ventral aspect of their neck and back shaved and scrubbed with betadine solution. Level of anesthesia is assessed by monitoring arterial pressure, corneal reflex, and hindlimb withdrawal to noxious stimuli. Using aseptic techniques, PE catheters are placed in the external carotid artery for blood pressure and heart rate monitoring, and in the external jugular vein for fluid and drug administration. Rats are orotracheally intubated with a PE 220 catheter, paralyzed with pancuronium bromide (0.6 mg i.v. followed by a 0.1 mg/kg/hr infusion), and artificially ventilated (Ugo Basile rodent respirator). An anesthetic regime of Halothane, oxygen, and air (see below) is adjusted to maintain physiologically normal levels of pO_2 and pCO_2 . Arterial blood gases are measured every hour with a blood gas analyzer (Radiometer ABL330; 75 μ l samples). Animals are paralyzed in order to: (1) fully control an animal's respiration and eliminate hypercarbic and hypoxemic effects of anesthetic agents; and (2) ensure physiologically normal blood gases and therefore mimic conditions as they occur in humans at the time of injury.

Contusive spinal cord injury: For producing traumatic spinal injury, a T-8 laminectomy is performed and animals positioned in the weight-drop apparatus as described by Noble and Wrathall (1987). The severity of injury is varied by adjusting the height of the weight drop (10g weight) as follows: mild injuries (5cm), moderate (12.5cm), and severe (25.0cm). As mentioned above the weight drop apparatus used was obtained from Dr. Wise Young and is presently the apparatus of choice in a multi-center trial evaluating the effects of drug treatment on acute spinal cord injury. This apparatus is accepted as producing reliable and reproducible injuries of mild, moderate or severe magnitude. The fact that it is being used in studies evaluating potential therapeutic interventions makes it an appropriate choice for use in the present study. Modifications to the parameters (weight and height) used for production of injuries were made based on evaluations of injuries. At the conclusion of surgery the incision is closed in layers, and catheters removed. Post-operatively, animals are housed in cages containing soft bedding and treated with cefazolin (40 mg) i.m. twice a day for 5 days. Water bottles were placed sufficiently low to allow access to water. Food was placed inside the cage until the rats are capable of reaching the standard placement in the cage top. Injured animals were checked daily and bladders palpated at least twice daily and emptied as required until they gain reflex voiding. Body weight was monitored weekly and records kept of all animal care. Antibiotics

were administered to animals exhibiting signs of urinary tract infection. Veterinary consultation was obtained for animals demonstrating discomfort or autonomy following injury. In our experience, autonomy was rare in animals with T8 spinal cord injuries. Inclusion criteria for animals to be used in the study consisted of: (a) paraplegia post weight drop; (b) spinal cord hematoma; (c) acceptable weight drop (compression, impact velocity, impact height); and (c) acceptable physiological parameters (blood pressure, pCO₂, blood pH).

Measurement and variation of temperatures: Systemic temperature is controlled with a temperature circulator connected to a cooling or warming blanket (Lauda RM6-6 unit which is accurate to 0.1 °C). A flexible thermistor probe (Physitemp IT-21, 410µm diameter) is inserted in the rectum to monitor systemic temperature, and a second thermistor placed laterally at the site of laminectomy in the epidural space to monitor epidural temperature.

Anesthetic Regime: The anesthetic regime consisted of Halothane (0.5-5.0%), nitrous oxide (20%) and oxygen (20%) in order to produce physiologically normal levels of pO₂ and pCO₂. The rationale for using this anesthetic combination is: (1) inhalational anesthetics provide a much easier induction, a more uniform level of anesthesia, and a more prompt recovery than injectable anesthetics such as barbiturates or ketamine; and (2) since halothane is commonly used in all studies carried out in The Miami Project, this anesthetic enables comparisons of results between studies.

During the first year of the funding period it should be noted that we made a modification in the anesthetic mixture given during surgery. The anesthetic regime consisted of isoflurane (0.5-5.0%), nitrous oxide (20%) and oxygen (20%) in order to produce physiologically normal levels of pO₂ and pCO₂. The change was made as it was noted that we experienced an unusually high mortality rate which was attributed to cardiovascular complications of halothane. In the second year the switch was made back to halothane after a thorough overhaul of our anesthetic and respiratory equipment was carried out. During the second year we did not experience any problems with regard to animal survival using halothane as an anesthetic.

Agmatine Administration: In experiments evaluating the effects of agmatine the drug was administered (I.P.) within 15 minutes after injury. After the initial injection, daily injections were given (at the same time each day) for a period of 14 days. Agmatine was dissolved in normal saline and administered at a dose of 100mg/kg. This injection protocol was used in experiments when agmatine was evaluated alone or in combination with hypothermia.

In all experiments each group of animals being evaluated consisted of a minimum of 5 animals. Statistical comparisons were carried out using analysis of variance (ANOVA). The volume of tissue damage, are presented as mean + standard error, and p-values of <0.05 were considered significant (see Appendix).

Histopathology of Experimental SCI: The morphological changes associated with experimental SCI have been documented in a variety of species (Allen, 1914, Ducker et al., 1971, Bresnahan et al., 1976, Balentine 1978a, 1978b, Noble and Wrathall, 1985). Hours following traumatic SCI hemorrhagic changes progress centrifugally and injured areas coalesce to form an area of hemorrhagic necrosis that extends along the longitudinal axis of the cord in a spindle shaped form (McVeigh, 1923, Ducker et al., 1971, Balentine, 1978a). The acute damage is located more centrally and, depending on the severity of the injury, it may progress to involve the adjacent white matter (Ducker et al., 1971). White matter changes begin in the areas adjacent to the gray matter and spread outward in a centrifugal fashion (Bresnahan et al., 1976, Bresnahan, 1978). By the end of the first week postinjury, demyelination and cystic degeneration of necrotic areas becomes evident, particularly in more severe injuries (Ducker et al., 1971, Blight, 1985). By four weeks, the cystic cavity is better defined and the surviving white matter displays demyelination and microcysts (Wagner et al., 1978, Noble and Wrathall, 1985). At four months the cyst is surrounded by astrocytic gliosis and the region of injury shows thickening of the dura mater. An increased cellularity of the leptomeninges is apparent, especially in the more severe injuries (Wagner et al.,

1978). Although the morphological analysis to be used in the present study was not extended four months, many of the same analytical protocols alluded to above will be used (see below).

Histological evaluation: At the termination of experiments, animals are deeply anesthetized with sodium pentobarbital and perfused transcardially with a solution of 4% paraformaldehyde and 3.6% glutaraldehyde in 0.1 M Sorensen's phosphate buffer. Injured cord segments, along with surrounding normal cord were removed.

(a) Data Analysis: An important aspect of all experiments is the quantification of results. In order to establish meaningful relationships among different treatment groups, it was imperative to quantify the amount of tissue damage for animals undergoing different treatments. To this end, transverse or horizontal sections were examined with light microscopy and preliminary reconstructions of the area of tissue damage, i.e. neuronal loss, axonal injury, were made with the aid of an overhead projector and camera lucida (using 1X or 4X objectives). This analysis was carried out by an individual "blinded" to the experimental design for tissue being analyzed. The area of maximal gray and white matter damage at the epicenter of injury sites was evaluated using computer aided image analysis (Image I, Universal Imaging Corp.). This technique has been used successfully in studies to quantify the amount of gray and white matter damage resulting from weight drop injury of the rat cord. This method involves the use of 20-30 longitudinal (horizontal) serial sections. In horizontal section the rostrocaudal boundaries of tissue damage can be found easily by evaluating the presence or absence of inflammatory cells, necrotic tissue, and macrophages. Using a low power (1X) objective camera lucida drawings are made of the gray matter of sections from the tissue block. Each area is then traced onto a digitizing tablet (Summagraphics) interfaced to a MicroVAX computer system, which computes areas at each horizontal level. The total necrotic area is derived by means of numerical integration of sequential areas. Based on results obtained during the past two years this method has provided an effective approach to quantitatively describing the region of tissue damage resulting from weight drop injury in the rat.

Behavioral Outcome: A number of methods have been devised for the assessment of residual neurological function following experimental SCI. The most widely utilized are modifications of Tarlov's score, which is an assessment of spontaneous locomotion (Tarlov, 1957), and the inclined plane score described by Rivlin and Tator (1977). Several other tests have been utilized, such as the response to paw pinch and reflex righting (Gale et al, 1985). Recently tests of sensory function including mechanical and thermal sensitivity (Hargreaves et al., 1988; Bennett and Xie, 1988; Hama and Sagen, 1993) have provided testing paradigms designed to evaluate the effects of injury on sensory systems. Although none of the above mentioned tests specifically address the integrity of individual spinal pathways, they do provide an index of the severity of injury and of an animal's overall neurological state. In our evaluation of behavioral recovery following hypothermia and/or agmatine we used the Basso-Beattie-Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). This scale is a multiple function test of locomotor outcome which provides an efficient, expanded and unambiguous locomotor rating (Basso et al., 1995). In the original proposal it was suggested that in addition to the BBB test that the following tests would also be used to assess neurological function: (1) inclined plane score - measuring the steepest angle a rat is able to maintain its position for at least five seconds; rats are tested with their heads facing right, left, up and down; (2) righting reflex - a measure of a rat's ability to return to the upright position after being placed flat on its back; in addition to hindlimb function this test also assesses trunk muscle function; (3) response to paw pinch - rats are scored on their response to paw pinch on a 0 (no response) to 4 (normal response) scale (a variant of this test using calibrated von Frey filaments to evaluate responses to mechanical stimuli can also be used); and (4) thermal paw flick - this test measures the response of an individual paw to varying intensities of thermal stimuli. Due to time constraints and because of the thorough evaluation achieved with the BBB, it was decided that tests 1-4 (above) would not be used.

Prior to using the BBB test pre-injury training sessions (two sessions for each rat) were carried out to familiarize the animals with the environment of the room in which they were tested and to get them used to being handled. Behavioral testing was carried out beginning on day 2 post-injury and was performed in a blinded fashion on days 2,5,9,12,16,19,23,26,30,33,37,40,44 post injury.

EXPERIMENTS CARRIED OUT DURING THE SECOND YEAR OF FUNDING

Experiment 1

Specific Aim: *Evaluate the effects of mild hypothermia on locomotor function following traumatic spinal cord injury.* The objective of these experiments was to study the effects of post traumatic hypothermia on behavioral outcome measures in animals subjected to traumatic SCI. This series of experiments combined results described in Experiments 1-4 of the original proposal.

Rational: During the first year it was demonstrated that mild hypothermia delivered for a period of 4 hours thirty minutes after injury resulted in significant neuroprotection following traumatic SCI. An important question related to this effect is whether there is any behavioral significance attached to this neuroprotective effect.

Experimental Protocol: Rats were anesthetized, intubated, paralyzed and artificially ventilated. Heart rate and blood pressure were monitored in all animals throughout the length of the experiment and pO₂ and pCO₂ maintained within normal physiologic limits. Blood gases were evaluated every 30 minutes. Systemic temperature was measured by a teflon thermocouple probe inserted in the rectum. Epidural temperature was measured with a teflon thermocouple flexible probe inserted under the dura. After a 30 minute control period following injury the epidural temperature was lowered by placing the animal in a plexiglas box with circulating thermal blanket. Systemic temperature was monitored continuously along with epidural temperature throughout a 4 hour period during which the epidural temperature was maintained a level of either 37°C (normothermic) or 32°C (hypothermic). Two days following these procedures all animals were evaluated for residual locomotor function using the BBB locomotor rating scale. This evaluation continued using the schedule described above for the duration of the survival period (44 days). At the conclusion of experiments animals were deeply anesthetized with sodium pentobarbital and following fixation, spinal cord tissue was removed and prepared for histological examination, including determination of the volume of tissue damage.

Results: The results of this study showed that there were significant differences between the locomotor scores of animals in the hypothermic versus normothermic groups (Figure 1). As shown in Figure 1 there was a dramatic increase in BBB scores between days 2-12 post-injury for animals in both experimental groups. Starting on test day 19, however, hypothermic animals began showing significant differences in their BBB scores when compared to animals in the normothermic group. At the conclusion of the evaluation period it should be pointed out that the BBB scores for normothermic animals were beginning a trend in the downward direction while those for the hypothermic animals were trending in an upward direction. These results not only support the functional benefits of hypothermia, but also indicate that these beneficial are long term.

In addition to evaluating locomotor scores during the survival period tissue was taken from all animals and subjected to a rigorous analysis of tissue damage. It was during this analysis that the first significant result was obtained that did not follow the logic and rationale of what might be expected from the effects of hypothermia. Contrary to what was reported in last years progress report there was no significant difference in the volume of tissue damage between the normothermic and hypothermic groups. This conclusion is based on the same type of volume analysis that was used during the first year. Needless to say, this result was quite surprising as one would predict a complementary morphological correlate to the behavioral results. At the present time efforts are being

made to reanalyze spinal cords from both groups of animals. The first group to be looked at consists of 5 normothermic and 5 hypothermic animals. In this reevaluation we are focusing on the possibility of a differential effect of hypothermia on gray versus white matter. The reason for this relates to the fact that the BBB score is closely related to the degree of damage in the white matter of the cord (Breshnahan, personal communication). Considering the significant effects of hypothermia on BBB scores, it is possible that this improvement is due largely to selective protection of ascending and descending white matter tracks and is less related to preservation of the gray matter. Once this analysis is complete we will commence with the analysis of an additional 15 cords from three groups of animals that were recently completed and which also showed significant differences between hypothermic and normothermic animals. In addition to a volume analysis of tissue damage we are also using a neurofilament stain as a way of evaluating changes in the white matter (e.g. axon profiles) of animals in different groups.

As stated above the results of the histological analysis of animals undergoing behavioral testing were surprising, but more importantly these results were also inconsistent with results reported last year. Another finding different from last year was the fact that the volume of tissue damaged was markedly less in recent animals compared with those done last year. As stated in the methods section (above) one major difference between animals evaluated in the first versus second year was a change in anesthetic (halothane vs. isoflurane). Although this has not been systemically evaluated it is possible that the damage observed in year one following hypothermia was in some way influenced by the isoflurane used in these animals. On the other hand, the use of halothane (used in year two) produced significantly less damage. At present we are not ready to draw any conclusions about these preliminary results as we feel additional animals must be evaluated. If the difference in the volume of tissue damage turns out to be due to an anesthetic effect this represents a significant finding for future clinical consideration and for the design of future experimental studies.

Experiment 2

Specific Aim: *Evaluate the effects of the systemic administration of agmatine on locomotor function following traumatic spinal cord injury.* The objective of this series of experiments was to study the effects of agmatine on morphological and behavioral outcome measures in animals undergoing traumatic SCI. This series of experiments is related to the objectives in Experiment 5 of the original proposal.

Rationale: Previously it has been shown that agmatine is neuroprotective in models of CNS injury (trauma and ischemia). Our own work has shown that agmatine administered systemically or intraspinally also produces significant neuroprotective effects against excitotoxic tissue damage produced by intraspinal injections of quisqualic acid. Considering the fact that agmatine is an NMDA antagonist and an inhibitor of NOS, we wanted to determine if agmatine could produce neuroprotective and/or behavioral effects following traumatic SCI. Based on our long term goals of combining therapeutic strategies with hypothermia it was also thought that this evaluation was an important first step towards accomplishing this goal with a substance that could be easily administered, i.e. systemically.

Experimental Protocol: Rats were anesthetized, intubated, paralyzed, and artificially ventilated. Heart rate and blood pressure were monitored throughout the length of experiments and pO₂ and pCO₂ maintained within normal physiologic range. Epidural temperature was maintained at the normothermic level of 37°C (systemic temperature 37°C). Epidural temperature was monitored with a flexible thermocouple probe and contusive lesions produced by the weight drop technique (NYU impactor). The time post-injury for the first injection of agmatine was 15 minutes. Animals received agmatine injections 100mg/kg every day for 14 days during the post injury survival period. At the conclusion of experiments animals were deeply anesthetized with sodium pentobarbital and following fixation, spinal cord tissue was removed and prepared for histological examination, including determination of the volume of tissue damage.

Results: The results of this study showed that agmatine produced significant differences in locomotor scores when compared to animals in the normothermic group (Figure 2). As shown in Figure 2 there was a significant improvement in BBB scores between days 2-9 post-injury for animals in the agmatine group. This initial improvement in agmatine animals is by itself an interesting observation and one that will require additional investigation. A comparable improvement in scores for animals in the normothermic group did not occur until day 16 post injury. Throughout the entire evaluation period the BBB scores for the agmatine animals were significantly higher for the agmatine group compared to animals in the normothermic group (Figure 2). An important point to keep in mind is that the agmatine treatment was only carried out until day 14 post-injury.

In addition to evaluating locomotor scores during the survival period tissue was taken from all animals and will be subjected to volume analysis of tissue damage. At the present time this tissue has not been analyzed as we are trying to decide the best way to proceed with this analysis. We have not begun this analysis as different staining protocols may require different handling of the tissue. This may be necessary depending on how we decide to analyze the tissue.

Experiment 3

Specific Aim: *Evaluate the effects of mild hypothermia plus the administration of agmatine on locomotor function following traumatic spinal cord injury.* The objective of these experiments was to evaluate the effects of post traumatic hypothermia plus systemically administered agmatine on behavioral outcome measures in animals subjected to traumatic SCI and epidural temperatures of 32°C. This series of experiments combines the results from Experiments 1-4 of the original proposal and addresses the objective in Experiment 5.

Rationale: Considering the positive effects of hypothermia or agmatine on behavioral outcome measures following traumatic SCI, the hypothesis was proposed that by combining these two interventions there may be a synergistic or additive effect of these two interventions.

Protocol: A protocol combining those described in Experiments 1 and 2 (above) was used in this study.

Results: The results of this combination therapy are shown in Figure 3. As shown there were significant effects at early and late time points for animals in the hypothermia + agmatine group compared to the BBB scores of animals in the normothermic group, but no evidence was observed of an additive or synergistic effect of these two interventions. This conclusion is based on the similarity of final BBB scores obtained in the hypothermia, agmatine and hypothermia + agmatine groups. A comparison of effects for the two interventions versus normothermia is shown in Figure 4. Although the results of this comparison don't show any advantage of combining hypothermia with agmatine one effect hypothermia may have is to extend the therapeutic window of agmatine. To test this hypothesis one would need to administer agmatine at different time points during the period of hypothermia and determine if similar results to agmatine administered at 30 minutes post injury would be obtained.

The results of this series of experiments leads to the conclusion that agmatine, which blocks NMDA receptors and inhibits NOS, is only able to provide behavioral results equal to that observed with hypothermia. Furthermore, it is suggested that the effects of agmatine and hypothermia are working through a comparable mechanism. For there to be an additive effect of pharmacological treatment and hypothermia it is suggested that a drug affecting another component of the secondary injury cascade must be used. One possibility is IL-10 which has been shown to produce neuroprotection in traumatic SCI by affecting the inflammatory component of the injury cascade (Bethea et al., 1998).

CONCLUSIONS AND FUTURE DIRECTIONS

During the second year of funding we determined that mild hypothermia (32° C) delivered within thirty minutes of a moderate injury for a period of four hours produces significant differences in locomotor scores when compared to normothermic animals. This result combines findings from the first year in which we determined the most effective duration of treatment, onset time, and injury severity required to produce neuroprotective effects of hypothermia. In a parallel series of experiments not directly related to the research plan of the hypothermia project we also determined that administration of the NMDA antagonist and NOS inhibitor agmatine also produces significant neuroprotective effects against excitotoxic injury resulting from the intraspinal injection of quisqualic acid. These effects were achieved with either intraspinal or systemic administration of agmatine. The encouraging results of this study led to a series of experiments closer to the hypothermia research plan and included an evaluation of agmatine effects against traumatic SCI. In these experiments we determined that the systemic administration of this drug produced significant behavioral effects. Because these effects could be achieved with systemic administration of the drug, unlike the effects of cytokines and growth factors evaluated in the first year which were obtained with intraspinal injection and our desire to combine hypothermia with a pharmacological intervention, the results with agmatine led us to the evaluation of combining hypothermia with the systemic administration of agmatine. Unfortunately, the combination of hypothermia and agmatine did not result in an additive or synergistic effect on behavioral outcome suggesting that hypothermia should be combined with an intervention directed at another component of the secondary injury cascade.

In the third year of funding there are four objectives that we would like to accomplish:

(a) Evaluate the effects of 8 hours of hypothermia commencing within 30 minutes of injury. To date our research has shown that mild hypothermia delivered for a period of 4 hours produces significant behavioral effects. To determine if a longer duration of hypothermic treatment is capable of producing an even greater morphological and behavioral effect we believe it is possible to carry out a study using the 8 hour time frame. Because of the need to keep animals anesthetized throughout the hypothermic period, it is felt that this is the upper limit that can be realistically evaluated. This study will address the objective described in Experiment 4 of the original proposal.

(b) One of the goals of the original research plan was to evaluate the effects of hyperthermia on morphological and behavioral outcome measures. To complete our evaluation of the effects of temperature on recovery from spinal cord injury, i.e., hypothermia and normothermia, we would like to evaluate the effects of hyperthermia on our morphological and behavioral outcome measures. This study will address an objective described in Experiment 2 of the original proposal.

(c) Recently it was shown that systemic administration of the anti-inflammatory cytokine IL-10 produces significant morphological and behavioral effects on a moderate injury of the spinal cord. Furthermore, no effects were found on more severe injury. In the first year of the funding period we determined that mild hypothermia by itself also did not produce any significant effects on a severe injury. Therefore, no interventions have been developed that result in beneficial effects on the most severe of injuries. Because of the clinical relevance of the severe injury, we believe it would be important to evaluate the effects of hypothermia and IL-10 on the severe injury. If successful this combination of interventions would offer a therapeutic strategy for any severity of spinal cord injury. We believe that this combination of effects is a reasonable combination, due to the fact that IL-10 produces its effect by effecting the inflammatory component of injury and because IL-10 is administered systemically. We will also evaluate the effects of the combination of these interventions on the moderate injury. This study combines results from Experiments 1-4 and addresses the objective described in Experiment 5 of the original proposal.

(d) The final objective is to try to determine if there are physiological correlates to the improved behavioral effects demonstrated during the past year. We believe, however, that it is also important to address the dilemma we have regarding the improved behavioral effects in a group of animals where there seemed to be no significant morphological effects. Resolving this dilemma will therefore be an important component of our research during the final year of funding and will focus on the evaluation of a differential effect of hypothermia on gray versus white matter in the cord. These studies address objectives outlined in Experiments 1-4 of the original proposal.

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APPENDICES

FIGURES:

FIGURE 1: Effects of mild hypothermia on locomotor function following traumatic spinal cord injury.

FIGURE 2: Effects of agmatine on locomotor function following traumatic spinal cord injury.

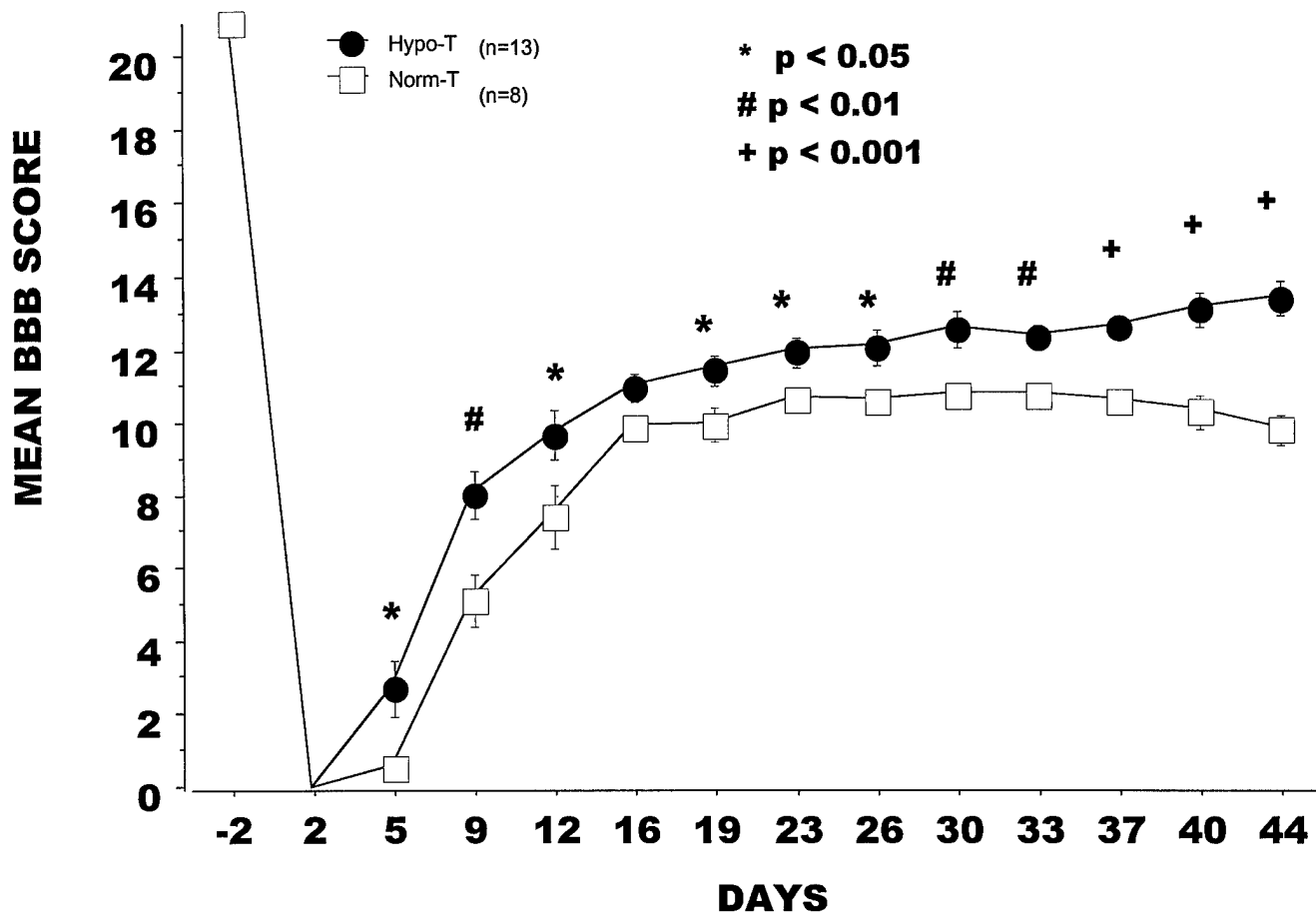
FIGURE 3: Effects of both agmatine and hypothermia on locomotor function following traumatic spinal cord injury.

FIGURE 4: Comparison of effects of hypothermia, hypothermia + agmatine and normothermia on locomotor function following traumatic spinal cord injury.

PAPERS:

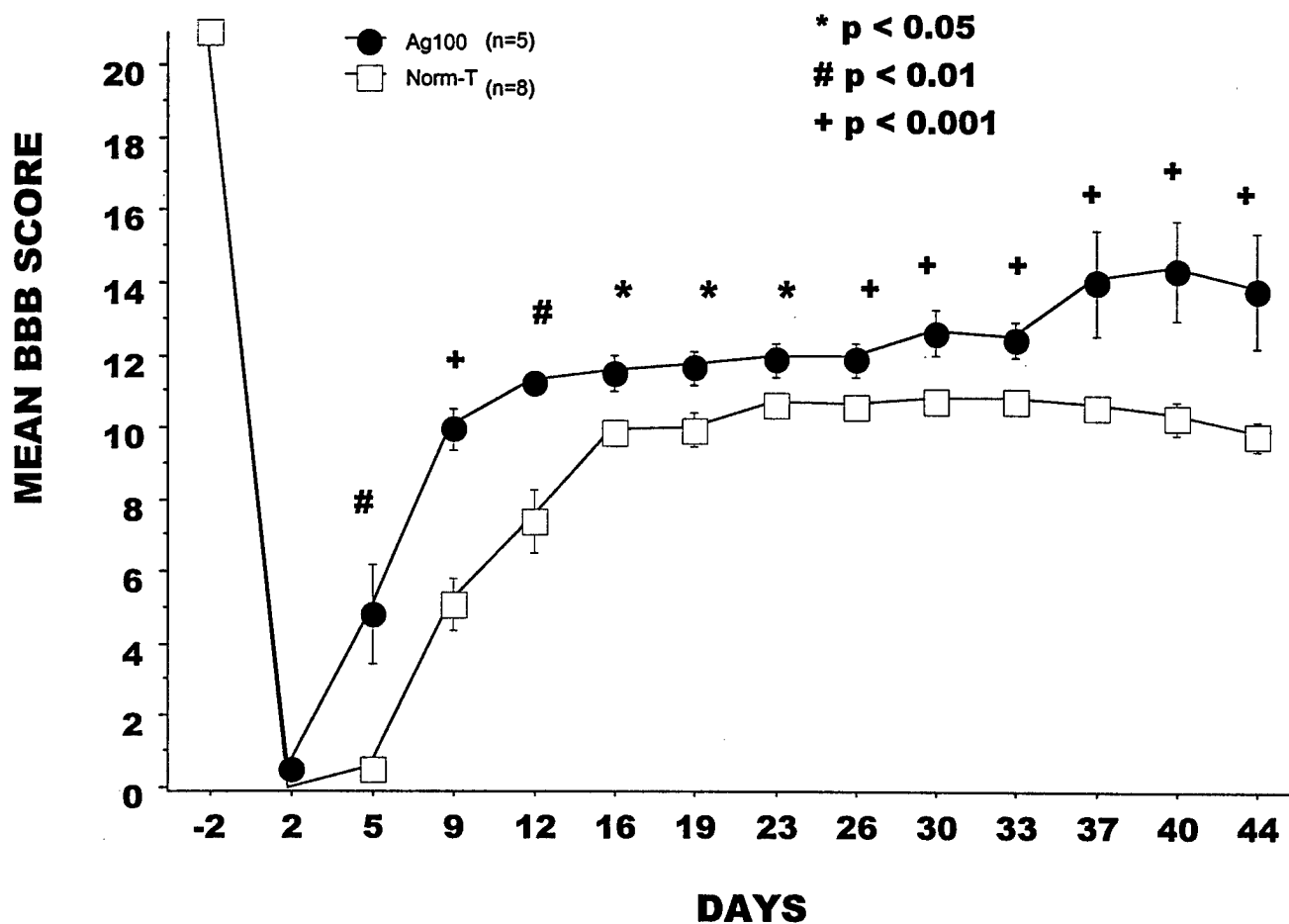
1. Manuscript describing the results of a study dealing with the neuroprotective effects of cytokines and neurotrophic factors: Lee et al. J. Neurotrauma (IN PRESS).

Manuscript describing the morphological and behavioral effects of systemic hypothermia on acute spinal cord injury is presently in preparation. This manuscript would have been complete if we had not experienced the unexpected result of a lack of morphological correlate to our behavioral effects.



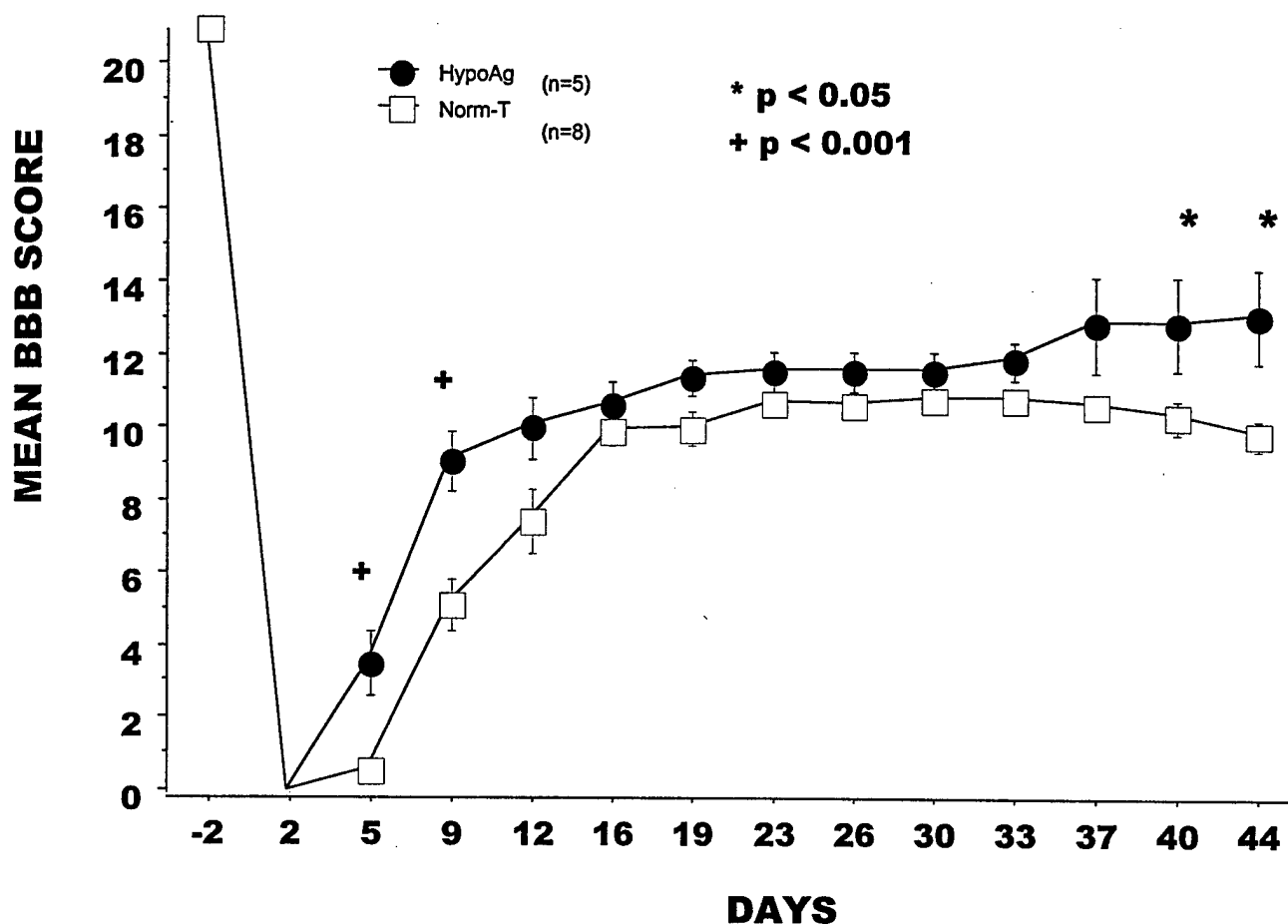
EFFECTS OF HYPOTHERMIA ON BBB

FIGURE 1: Effects of mild hypothermia on locomotor scores following traumatic spinal cord injury. Animals received four hours of hypothermia (32°C) commencing 30 minutes after injury. Beginning on day two post-injury and continuing until day 44 animals were evaluated using the locomotor rating scale of Brasso-Beattie-Breshnahan (BBB). Mean behavioral scores for animals receiving hypothermic (●) versus normothermic (37°C) treatment (□) are shown on the y-axis and days post-injury on the x-axis. P-values obtained from the comparison of the two treatments are indicated.



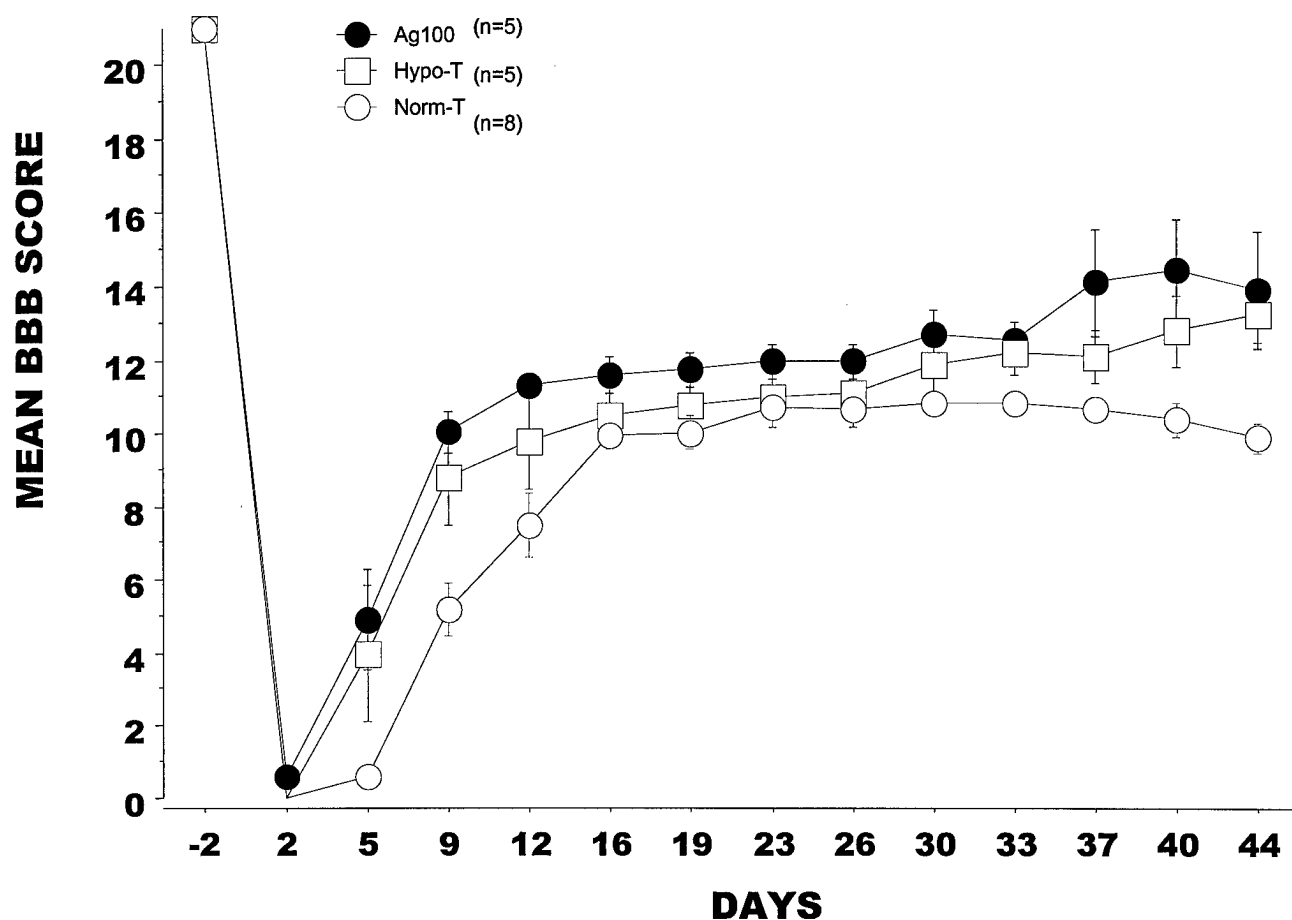
EFFECTS OF AGMATINE ON BBB

FIGURE 2: Effects of agmatine on locomotor scores following traumatic spinal cord injury. Animals received the first injection of agmatine 30 minutes post-injury (100mg/kg). Daily injections of agmatine were given for 14 days post-injury. Beginning on day two post-injury and continuing until day 44 animals were evaluated using the locomotor rating scale of Brasso-Beattie-Breshnahan (BBB). Mean behavioral scores for animals receiving agmatine (●) versus normothermic (37°C) treatment (□) are shown on the y-axis and days post-injury on the x-axis. P-values obtained from the comparison of the two treatments are indicated.



EFFECTS OF HYPOTHERMIA WITH AGMATINE ON BBB

FIGURE 3: Effects of mild hypothermia combined with systemic injections of agmatine on locomotor scores following traumatic spinal cord injury. Animals received four hours of hypothermia (32°C) commencing 30 minutes after injury. Animals also received the first injection of agmatine (100mg/kg) at this time and daily for 14 days post injury. Beginning on day two post-injury and continuing until day 44 animals were evaluated using the locomotor rating scale of Brasso-Beattie-Breshnahan (BBB). Mean behavioral scores for animals receiving hypothermic + agmatine (●) versus normothermic (37°C) treatment (□) are shown on the y-axis and days post-injury on the x-axis. P-values obtained from the comparison of the two treatments are indicated.



EFFECTS OF HYPOTHERMIA AND AGMATINE ON BBB

FIGURE 4: Comparison of three treatment protocols against locomotor scores following traumatic spinal cord injury. Effects of agmatine (●), mild hypothermia (□), and hypothermia combined with systemic injections of agmatine (○) on locomotor scores following traumatic spinal cord injury. Animals received four hours of hypothermia (32°C) commencing 30 minutes after injury. Animals received the first injection of agmatine (100mg/kg) at this time and daily for 14 days post injury. Beginning on day two post-injury and continuing until day 44 animals were evaluated using the locomotor rating scale of Brasso-Beattie-Breshnahan (BBB). Mean behavioral scores for animals receiving the different treatments are shown on the y-axis and days post-injury on the x-axis. Note that the most significant differences between the hypothermic and agmatine treatment groups versus the normothermic group occurs at the beginning and end of the survival period.

Neuroprotective Effects of Basic Fibroblast Growth Factor (bFGF) Following Spinal Cord Contusion Injury in the Rat

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Running Head: Neuroprotection Following Spinal Contusion

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ABSTRACT

Cytokines and neurotrophic factors have been implicated in the pathophysiology of injury to the central nervous system. While some cytokines are considered pro-inflammatory, other factors promote neuronal growth and survival. The present study investigated the neuroprotective effects of interleukins 1 (IL-1), 4 (IL-4), and 6 (IL-6), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF) in a contusion model of spinal cord injury. Female Sprague-Dawley rats (n=55) sustained a 10-gram weight drop injury to the lower thoracic spinal cord (T10) from a height of 12.5mm using the NYU impactor. A micro-infusion system (Alzet minipump) was used to continuously deliver drugs or vehicle directly into the epicenter of the contused spinal cord starting one or three hours post-injury. At the end of 7 days, animals were perfused and the cords removed for histopathological analysis. Longitudinal serial sections were cut on a freezing microtome and stained with cresyl violet. Areas of central necrosis, partial preservation, and total zone of tissue injury were identified and traced by an independent reviewer using a computer based imaging system. The mean total zone of injury in 5 animals receiving vehicle infusion was $18.04 \pm 1.88 \text{ mm}^3$. The mean zone of partial preservation in these animals was $16.46 \pm 1.49 \text{ mm}^3$. Basic fibroblast growth factor reduced the total zone of injury by 33% [$p < 0.01$, least significant difference (LSD) of Fisher] in 5 animals and the zone of partial preservation by 32% ($p < 0.01$, LSD of Fisher) when compared to controls. Interleukin-4, NGF, and CNTF reduced the total zone of injury (LSD of Fisher: IL-4, $p = 0.12$; CNTF, $p = 0.10$; NGF, $p = 0.13$), as well as the zone of partial preservation (LSD of Fisher: IL-4, $p = 0.18$; CNTF, $p = 0.24$; NGF, $p = 0.18$), though none of the changes were statistically significant. No significant differences were observed between animals receiving vehicle versus bFGF treatment commencing 3 hours after injury. These data demonstrate that the continuous intramedullary infusion of bFGF initiated one hour after moderate contusion injury of the spinal cord significantly reduces the total zone of injury and the zone of partial preservation. These results support the further investigation and possible future clinical application of bFGF in the treatment of acute spinal cord contusion injury.

INTRODUCTION

The initial trauma induced by injury together with a complex cascade of events following injury determine the degree of total tissue damage and the ultimate neurological outcome following spinal cord injury (SCI). Presently, numerous agents are proposed to be neuroprotective against CNS injury (for recent reviews see McIntosh, 1993; Mattson and Scheff, 1994; Moccetti and Wrathall, 1995). Steroids, neurotrophins, cytokines, and gangliosides have been demonstrated to promote neuronal survival or support neuronal growth in various *in vivo* systems (Mattson and Scheff, 1994; Blottner and Baumbarten, 1994; Olson et al. 1994.). Methylprednisolone improves neurological recovery when given early after human spinal cord injury (Bracken et al., 1990). Recently, neurotrophins have also been used in several disease models: glial cell-derived neurotrophic factor (GDNF) in Parkinson's disease, nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) in Alzheimer's disease, and insulin-like growth factor (IGF-1) in multiple sclerosis (Hefti, 1997). The neurotrophic factor basic fibroblast growth factor (bFGF) has also been reported to be neuroprotective in models of cerebral ischemia and traumatic brain injury (Koketsu et al., 1994; Fisher et al., 1995; Dietrich et al. 1996; Ay and Finklestein, 1998) and to protect neurons from axotomy-induced death (Peterson et al., 1996). In a model of spinal cord compression, bFGF administered locally at the site of lesion was reported to improve hindlimb function in combination with methylprednisolone infusion (Baffour et al., 1995). Recently Teng et al. (1997) reported basic and acidic FGF to be neuroprotective for cholinergic neurons following contusion injury in the rat.

IL-4 has been shown to be an anti-inflammatory cytokine, regulating neutrophil and monocyte/macrophage functions (Luering et al. 1997; Niirio et al. 1997). Although the clinical significance has not been established, increased levels of IL-6 have been reported in the cerebrospinal fluid of head injury patients (Relton et al. 1997). IL-1, a pro-inflammatory cytokine, was found to exacerbate ischemic brain injury (Relton et al. 1997). In the study of spinal cord trauma, experimental studies are needed to assess the consequences of neurotrophic growth factor and cytokine treatment on histopathological outcome.

The purpose of the present study was to determine the effects of various cytokines and neurotrophic factors on histopathological outcome using a well characterized weight-drop device to produce spinal cord trauma. In this preliminary examination of different agents we utilized a continuous intramedullary infusion system to reliably deliver various factors directly into the site of injury. Our results indicate that the intramedullary infusion of bFGF initiated one hour after moderate SCI significantly reduces two measures of tissue damage, including the zone of partial preservation and the total zone of injury.

MATERIALS AND METHODS

Model of Injury: Adult female Sprague Dawley rats weighing 250-325gms were used in this study. All procedures were approved by the University of Miami Animal Care and Use Committee. Inhalational anesthesia was provided with a balanced halothane, NO₂ and O₂ mixture. Local anesthetic of 0.25% lidocaine with 1:400000 epinephrine was used to infiltrate the skin and paraspinous muscles. A single dose of intramuscular antibiotic (50mg/kg of cefozalin) was administered at the beginning of the procedure. Aseptic techniques were employed to perform a one level complete laminectomy with bilateral medial facetectomy at the lower thoracic (T10) level of the cord. A 10gm weight drop utilizing the NYU impactor from 12.5mm was performed while monitoring start time, height, and velocity curves. Weight drops with less than 5% height and velocity errors, as well as resultant bilateral cord hematoma and immediate postoperative paraplegia were used as inclusion criteria. Rectal temperature was monitored and isothermic blankets utilized to maintain core temperature at 37°C during the surgical procedure.

Cytokines/growth factors: All drugs were purchased from Genzyme Corporation (Cambridge, MA), including interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and ciliary neurotrophic factor (CNTF). Solvents and dilution factors used to prepare each drug for infusion are listed in Table 1. Two assumptions were made in the calculation of infused drug concentration to ensure adequate drug delivery to the different zones of injury: 1) the cord is approximately a cylinder ($V = \pi r^2 h$) with an average cord diameter of 0.4cm at the T10 level; and 2) there is a 1:10 dilutional effect by cerebrospinal fluid. In preliminary experiments it was determined

with the infusion of 2% Evans Blue for seven days after weight drop (data not shown) that the longitudinal lesion length for the injury used in the present study was 0.8cm. Based on this determination the total cord volume to be infused was approximately 0.1cc³. The amount of drug calculated for infusion was delivered every day until sacrifice. A 2-10 times excess (based on previously tested *in vitro* concentrations reported by Genzyme Corporation) infusion was achieved for each factor (Table 1). For the purpose of evaluating the vehicle effect of different solvents used with each factor we choose a solution of saline and 0.1M PBS with 0.5% BSA for infusion in vehicle-treated animals. No efforts were made to evaluate the effects of H₂O or 0.1% CHAPS + 0.5% BSA in control animals.

Drug Delivery: An osmotically-driven infusion pump (*Alzet minipump*, Alza Pharmaceuticals, Palo Alto, CA) was used for continuous infusion of drugs. The pump was connected with PE10/50 tubing to a 30-gauge 3/4 inch needle to deliver drugs through a small dural and pial opening at the site of injury. The needle was advanced 2mm near the midline at the site of contusion. Needle insertion and infusion of drugs were started one hour post injury, and continued at 1ul/hr for 7 days. The *Alzet* minipump was pre-primed and loaded with drugs, and filled to 225ul, including the extra tubing (5cm). The injection cannula was secured to the preserved lamina below the level of injury and the pump was secured in a subcutaneous pocket with sutures.

Assessment of injury and protection: Animals were perfused with 10% formalin on day seven under sodium pentobarbital anesthesia. After fixation cords were removed and placed in 10% sucrose for 24 hours. Cords were cut longitudinally (100um) on a freezing microtome and stained with cresyl violet. Two-dimensional mapping of the injury site was carried out. Area values were then used to calculate the volume of injury in each experimental group by numeric integration of sequential areas. In preliminary experiments designed to evaluate the reproducibility of the injury protocol, the NYU impactor was shown to produce a reproducible area of spinal cord contusion at 7 days after injury. The well demarcated injury site consisted of an area of central necrosis (CN) defined as the area of cystic degeneration and complete tissue necrosis (Fig. 1). Surrounding the area of CN was commonly a zone that appeared partially preserved. This zone of partial preservation (ZPP) was defined as the region of vacuolation, selective

neuronal injury, and white matter swelling (Fig. 1). The present 3-zone injury model was established to recognize the total impact of the injury (TZI), the irreversible zone of injury (CN), and the intermediate injury zone which could potentially be rescued and reduced with treatment (ZPP). In this study, both the area of central necrosis and total zone of injury (TZI) were measured by the same independent blinded reviewer. The zone of partial preservation was calculated as the difference between TZI and CN (Fig. 1).

Experimental and vehicle-treated groups consisted of 7-8 rats. In all groups except the IL-1 treated animals, no more than one animal died prior to perfusion in each group. In the IL-1 treated animals, 5 of 12 animals died prior to perfusion. The cords of 7 animals were processed in each group, and the 2 animals with the highest and lowest volumes (TZI) were excluded from analysis. Experimental groups consisting of 5 animals were compared with vehicle-treated SCI animals ($n=5$). The data were analyzed first with the Shapiro-Wilk test to confirm the normal distribution of each data set (Shapiro and Wilk, 1965). The results of this test dictated the use of parametric statistics and therefore a one-way ANOVA was used to determine that there were significant differences among the different experimental groups. To determine which groups were different, we then performed the least significant difference procedure of Fisher (Daniel, 1995). The volume of tissue damage, i.e. TZI and ZPP, are presented as mean \pm standard error, and p-values of <0.05 were considered significant.

RESULTS

In the vehicle-treated group ($n=5$), the mean volume of TZI was $18.04 \pm 1.88\text{mm}^3$ and the mean ZPP was $16.46 \pm 1.49\text{mm}^3$. The mean injury volumes of different treatment groups are listed in Table 2. Animals receiving interleukin-1 (IL-1) were found, on the average, to have larger TZI and ZPP volumes compared to control animals, though the difference was not significant ($p > .05$). The animal survival rate was also lower (7/12) in the IL-1-treated group. Interleukin 6 (IL-6) did not have any appreciable effects on any of the injury parameters (Figs. 2,3). By contrast basic fibroblast growth factor (bFGF) was the most effective agent in reducing both the TZI ($p < 0.01$, LSD of Fisher), and ZPP ($p < = 0.01$, LSD of Fisher)

(Figs. 2,3). Overall, bFGF reduced TZI by 33% and ZPP by 32% compared to control animals. The effect on central necrosis was more variable, and not statistically significant.

Though not statistically significant, three other drugs were also found to have effects on the volume of contusion injury. Ciliary neurotrophic factor, NGF and IL-4 reduced TZI (LSD of Fisher: CNTF, $p=0.10$; NGF, $p=0.13$; IL-4, $p=0.12$). CNTF infusion had the highest percentage reduction (23%) of these three factors on TZI (IL-4: 22%; NGF: 21%). Of these three factors, IL-4 reduced ZPP by 20% below control level, CNTF (17%). None of the drugs tested reduced central necrosis to a statistically significant level because of variability and the relatively small volumes of central necrosis, though CNTF, IL-6, and NGF did show a sizable mean percentage reduction of over 80% (Table 2).

In an effort to evaluate the potential therapeutic window of drug infusion, bFGF as the most effective treatment was selected for delivery three hours post injury. The comparison of this treatment with vehicle infusion showed no significant reduction in either TZI or ZPP (Fig. 4) ($p > 0.05$, LSD of Fisher).

DISCUSSION

The CNS response to injury uniquely involves the interactions between multiple cell types and injury processes (Hefti, 1997; Hirschberg et al. 1994; Relton et al. 1997). Cytokines and neurotrophic factors have been reported to promote neural growth and protect against ischemic, traumatic, and chemically-induced neuronal damage. These intercellular factors act primarily in a paracrine fashion, with glial and inflammatory cells producing and secreting them locally (Maeda, 1994). The post-traumatic breakdown of the blood-brain-barrier (BBB) also permits the extravasation of blood-borne factors and cellular elements that would be expected to promote inflammatory processes. Thus, two primary factors may potentially act against each other following the initial damage of the central nervous system: a) inflammatory factors which exacerbate damage; and b) the cytokines/neurotrophic factors which promote neuronal regeneration and recovery (Giulian et al. 1988). Certain cytokines may cause further tissue damage by the induction of a surface mitogen mediated immune response, as well as by direct cytotoxicity (Birdsall, 1991). It must be stressed that following injury to the brain or spinal cord, multiple factors affect

the cellular response to injury, leading to complex interactions that may affect outcome. Thus, although the use of *in vitro* purified cellular systems are a powerful approach in which to investigate the cellular response to injury, experimental models of CNS injury are necessary to determine the potential use of these agents in a clinical setting.

The weight drop system, utilizing the NYU impactor, provides a well characterized model for spinal cord injury. Most spinal cord injuries are blunt in nature, causing parenchymal contusions, hematoma, and edema. In the present study, evidence of a well-defined contusion was seen at seven days after injury. The injury volumes used in the present study consisted of the total zone of injury (TZI), zone of partial preservation (ZPP), and central necrosis (CN). This designation distinguishes between the areas of partial injury, which could be potentially reduced, and the zone of total destruction caused by the initial impact. Furthermore, this method of analysis allows for the testing of different therapeutic strategies against different areas of injury using the technique of quantitative image analysis.

Although the utilization of an injection needle in the present study to deliver putative therapeutic agents was invasive, this method assured the direct delivery of the various factors to the injury site. Based on previous data from brain injury studies (Fisher et al., 1995; Dietrich et al., 1997), intravenous infusions may also be considered as a method of drug delivery in the future. Potential problems including systemic side effects or unpredictable rates of drug delivery to the injury site may limit this method of drug delivery. However, systemic infusion is certainly the easiest in terms of clinical application, and has been used with bFGF in a clinical trial for acute stroke (Finklestein, personal communication). Intrathecal infusion remains another possibility, although site-directed drug delivery is not achieved with this approach.

Both interleukin-1 (IL-1) and tumor necrosis factor (TNF) are primarily the products of monocyte/macrophage/microglia lineage of cells. IL-1 stimulates monocytes and macrophages in an autocrine and paracrine fashion. T and B cell activation is another function of IL-1. IL-1 and its receptors have been widely characterized and mapped in the brain (Yabuuchi et al., 1994), and appear to play a significant role in thermoregulation. IL-1 may also regulate neuron-glial, and glial-glial interactions (Hannum et al. 1991). Significant elevations in IL-1 have been demonstrated after experimental ischemia

and trauma investigations and following clinical head injury (Relton et al. 1997). In addition, some benefits were reported after brain injury with the administration of the recombinant IL-1 receptor antagonists (Hannum et al. 1990; Relton et al. 1997). IL-1 has been reported to induce intercellular adhesion molecule 1 (ICAM-1) expression and neutrophil-mediated immune responses (Birdsall, 1991). In the present study, a detrimental effect of IL-1 on contusion volume was observed (Figs. 2-3), though the difference was not statistically significant. The administration of neutral antibodies directed against IL-1, or corresponding receptors may still prove beneficial in the treatment of acute SCI.

Interleukin-3 (IL-3) and interleukin-4 (IL-4) are T-lymphocyte derivatives and primarily activate B cells (Lee et al, 1993). Both cytokines have been reported to stimulate peripheral monocyte and microglia growth, and activate ICAM-1 and lymphocyte function associated antigen (LFA-1) on microglial surfaces (Lee et al., 1993b). Possible surface mitogen-mediated cellular and humoral immunity may ensue. IL-4 has been observed to downregulate monocytes and neutrophils (Lugering et al. 1997; Niiro et al. 1997). Their direct effects on neurons have not been fully characterized. In the present study, IL-4 reduced TZI by 22% and ZPP by 20%, though the reduction was not statistically significant possibly due to the small sample size. Considering the neuroprotective effects of the potent anti-inflammatory agent IL-10 (Bethea et al., 1998; Brewer et al., 1998), the anti-inflammatory effects of IL-4 may have accounted for some of the reduction of injury volumes in the present study (Figs. 2-3).

Interleukin-6 (IL-6) is produced by monocytes, macrophages, fibroblasts, activated T and B cells, and astrocytes *in vitro* (Lee et al. 1993). The target cells include pleuri-potential progenitor, B cells, and cytotoxic T cells. IL-6 has been reported to sustain both astrocyte and neuron survival *in vitro* (Kushima and Hatanaka, 1992; Maeda et al. 1994), and appears to interact with a subunit of the ciliary neurotrophic factor (CNTF) receptor (Saad, 1991). Generally considered to be pro-inflammatory, IL-6 has been reported to promote the survival of acetylcholinesterase (AChE) positive neurons in embryonic rat spinal cord cultures (Kushima and Hatanaka, 1992) and enhance neuronal survival from hypoxia/reoxygenation injury (Maeda et al. 1994). Increased cerebrospinal cord levels of IL-6 have been observed in both adult and pediatric head injured patients (Bell et al. 1997; Relton et al. 1997). Ras-GTP complex accumulation

in pheochromocytoma cell lines was induced by IL-6 as well (Nakafuku et al. 1992). IL-6 was shown in the present study not to be neuroprotective after contusion injury.

Both neurotrophins NGF (Blottner and Baumbarten, 1994; Holtzman et al. 1996; Oudega and Hagg, 1996; Saad et al. 1991, Sariola et al. 1994) and CNTF (Blottner and Baumbarten, 1994; Hefti, 1997) trigger neuronal regeneration and induce neuronal differentiation. NGF protects against ischemic brain injury *in vitro* (Holtzman et al. 1996). Its protective action for cholinergic neurons has also been demonstrated (Quirion et al. 1991). The CNTF_a receptor is homologous to the IL-6 receptor (Sariola et al. 1994) in that similar dimerization mechanism of their receptors have been reported. Both have been utilized for the experimental treatment of neurodegenerative disease with variable results (Blottner and Baumbarten, 1994; Hefti, 1997). In this study, both CNTF and NGF reduced TZI (23.2% and 21.3%, respectively), as well as ZPP (17.1% and 19.6%, respectively). The difference was not statistically significant possibly due to the small sample size.

Fibroblast growth factor (FGF) stimulates neuronal proliferation and sustains survival (Hefti, 1997; Murphy et al, 1994; Olson, 1994), but apparently inhibits differentiation (Murphy et al. 1994). FGF also activates rat pheochromocytoma PC12 cells (Nakafuku et al. 1992), and has been reported to enhance the growth of fetal cerebral cortex, hippocampus, and spinal cord neurons (Olson et al. 1994). FGF is a powerful stimulator of angiogenesis, and promotes wound healing. Recently, Cheng et al. (1996) successfully utilized acidic FGF (aFGF, FGF-1) as part of the growth medium for nerve grafts after rat thoracic spinal cord transection. Basic FGF or FGF-2 has been reported to be neuroprotective following experimental focal ischemia and traumatic brain injury (Fisher et al., 1995; Dietrich et al. 1996; Ay and Finklestein, 1998). In recent studies, bFGF together with methylprednisolone treatment was also found to improve behavioral recovery after spinal cord compression (Baffour et al., 1995) and both bFGF and aFGF protect cholinergic neurons following contusion SCI (Teng et al., 1997). bFGF stimulates astrocyte proliferation, and may mediate glial and neuronal interactions important to cell survival (Giulian, 1988). In the present study, bFGF was found to significantly reduce both the total zone of injury and the zone of

partial preservation. Based on these and previous findings, we conclude that bFGF is an important candidate for future investigations directed towards establishing a therapy for acute SCI.

Future studies that should be considered in developing a therapeutic strategy for SCI include combining various factors such as bFGF, IL-4, and CNTF with other treatment modalities. For example, Balfour and colleagues (1995) have reported a synergistic effect of bFGF and methylprednisolone on neurological function after experimental SCI, and the recent success of moderate hypothermia in the clinical treatment of traumatic brain injury (Marion et al. 1997) and in experimental studies (Martinez-Arizala and Green, 1992; Dietrich et al., 1994; Jiminez et al., 1997) suggests the combined use of mild hypothermia with infusion of bFGF. In the present study bFGF delivered three hours after injury did not exhibit neuroprotection, but further experiments to better delineate the therapeutic window for bFGF treatment need to be performed. Finally, preliminary experiments with intravenous bFGF infusion following contusion SCI have shown favorable results (Dietrich et al., unpublished observations) and this route of administration needs further investigation in studies with long-term survivals and behavioral testing to fully evaluate the benefits of this treatment paradigm. Based on the results of the present study combined with other clinical and pre-clinical data, it is proposed that the use of nerve growth factors, together with anti-inflammatory cytokines should be considered for future application in the treatment of acute SCI.

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FIGURE LEGENDS

FIGURE 1 Schematic illustration of the spinal cord contusion injury caused by the NYU impactor. TZI = total zone of injury; ZPP = zone of partial preservation; CN = central necrosis. Central necrosis was defined as the area of cystic degeneration and tissue necrosis. The zone of partial preservation was defined as the region of vacuolation, selective neuronal injury, and parenchymal edema, though the neuropil was intact and was calculated as the difference between TZI and CN.

FIGURE 2: Effects of drug infusion commencing one hour post injury and continuing for seven days on the mean volume for the total zone of injury (TZI). Compared to infusion of vehicle (control) IL-1 was found to have a detrimental effect, IL-6 no effect while bFGF significantly reduced the TZI. Although the effects of CNTF, NGF, and IL-4 were not significant they did produce a reduction in the volume of TZI. ** = $p < 0.01$.

FIGURE 3: Effects of drug infusion commencing one hour post injury and continuing for seven days on the mean volume for the zone of partial preservation (ZPP). Compared to infusion of vehicle (control) IL-1 and IL-6 were found to have detrimental effects while bFGF significantly reduced the ZPP. Although the effects of CNTF, NGF, and IL-4 were not significant they did produce a reduction in the volume of ZPP. ** = $p < 0.01$.

FIGURE 4: Effects of bFGF infusion commencing three hours post injury and continuing for seven days. No significant difference was observed between animals infused with vehicle and those receiving bFGF on either the total zone of injury (TZI) or zone of partial preservation (ZPP).

FIGURE 5: Sections taken through the epicenter of the weight drop injury after infusion of vehicle (A,B) or bFGF (C,D). Note the large area of damaged gray and white matter in the vehicle-treated cord. By contrast, the area of gray matter damage in the bFGF cord is smaller and there is increased preservation of white matter. The area of total injury (white and gray matter) is outlined with arrows in A and C. Scale bar in D equals 915 μ m (A,C) and 355 μ m (B,D).

Table 1: Dosage of interleukins and growth factors. The recommended *in vitro* concentrations were published by Genzyme Corporation.

Factor	Vial []	Sp activity	Solvent	Dil	[Final]	Exc	Lit. []
IL-1 _a	25 ug/ml	8x10 ⁶ u/mg	PBS + 0.1% BSA	1:100	2x10 ³ u/ml	5x	10U/ml
IL-4	1.5ug/ml	5x10 ⁸ u/mg	PBS + 1% BSA	No	1.5x10 ⁴ u/ml	2x	200U/ml
IL-6	5 ug/ml	5x10 ⁸ u/mg	PBS + 0.1% BSA	1:20	1.25x10 ⁵ u/ml	6x	500U/ml
NGF	20ug/ml	5x10 ⁵ u/mg	H ₂ O	No	2x10 ⁴ ng/ml	2x	100ng/ml
CNTF	100ug/ml	2x10 ⁷ u/mg	PBS + 0.1% BSA	1:20	5x10 ³ ng/ml	12x	10ng/ml
bFGF	1mg/ml	N/A	0.1% CHAPS 0.5% BSA	1:500	10 ⁴ ng/ml	2x	20ng/ml

Table 2. Effect of cytokines and growth factors on spinal cord contusion injury. ZPP was calculated as TZI - CN.

Drug Infusion	Total Zone of Injury	Central Necrosis	Zone of partial preservation
Control	$18.04 \pm 1.88 \text{ mm}^3$	$1.57 \pm 2.08 \text{ mm}^3$	$16.46 \pm 1.49 \text{ mm}^3$
IL-6	$18.03 \pm 1.19 \text{ mm}^3$	$0.42 \pm 0.37 \text{ mm}^3$	$17.61 \pm 1.28 \text{ mm}^3$
IL-1	$21.43 \pm 2.89 \text{ mm}^3$	$1.01 \pm 1.93 \text{ mm}^3$	$20.42 \pm 2.98 \text{ mm}^3$
IL-4	$14.07 \pm 0.91 \text{ mm}^3$	$0.89 \pm 1.08 \text{ mm}^3$	$13.18 \pm 1.36 \text{ mm}^3$
bFGF	$12.05 \pm 1.38 \text{ mm}^3$	$1.09 \pm 0.58 \text{ mm}^3$	$11.17 \pm 1.21 \text{ mm}^3$
NGF	$14.20 \pm 2.06 \text{ mm}^3$	$0.89 \pm 1.45 \text{ mm}^3$	$13.23 \pm 1.66 \text{ mm}^3$
CNTF	$13.86 \pm 1.10 \text{ mm}^3$	$0.22 \pm 0.28 \text{ mm}^3$	$13.64 \pm 0.99 \text{ mm}^3$

Figure 1. Central necrosis (CN) was defined as area of cystic degeneration and frank tissue necrosis (1a). The zone of partial preservation (ZPP) was defined as region of vacuolation, selective neuronal injury, and parenchymal edema, though the neuropil was intact (1b). Both the central necrosis and the total zone of injury (TZI) were measured by an independent blinded reviewer. Schematic illustration (1c) of spinal cord contusion injury caused by the impactor. $TZI = ZPP + CN$.

